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Patentanmeldung Nr. Patent application No. Demande de brevet n°

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PRIORITY DOCUMENT

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DSM N.V.

A NEW TEST METHOD FOR THE DETECTION OF ANTIMICROBIAL RESIDUES IN LIQUIDS

Field of the invention

The present invention relates to a novel method for the rapid detection of the presence or absence of antimicrobial residues in products preferably food products. A one step test method is described in which residues of antimicrobial compounds such as antibiotics are detected while disturbing compounds such as natural pigments (e.g. blood) are naturally present in the samples (e.g. lysozyme), and which may interfere with the test.

Description of the prior art

The presence of antimicrobial residues in food and feed is a growing concern among the consumers due to health-related problems and the increase of drug resistant bacteria. Antibiotics are not only applied as medication, but also widely used as antimicrobial growth promoting substances.

Antimicrobial residues might be present in e.g. body liquids, organs, meat and eggs which are used for consumption. Antimicrobial residues might also be present in food products in which said animal products are added as an ingredient. Examples of food products are milk; meat of cow, pig, poultry and fish; sea food such as shrimps; liver; processed meat products such as sausages; ready to eat meals and baby food. Antimicrobial residues might also be present in body liquids or animal tissues, which are suitable for examination by for example food-inspection authorities. Examples are blood, kidney tissue or pre-urine obtained from

the kidney and urine. Urine and blood are suitable for examination prior to slaughtering of the animal.

It is well known that animal body liquids, animal tissues and food products may contain too high concentrations of antimicrobial residues. In most countries, such as the countries of the European Union, Canada and the United States, Maximum Residue Levels (MRL) are regulated by legislation.

Test methods to detect antimicrobial residues in milk products such as microbial inhibition tests (e.g. agar diffusion tests) or methods making use of selective binders (e.g. antibodies or tracers) have been known for a long time. Examples of microbiological test methods have been described in GB-A-1467439, EP 0005891, DE 3613794, CA 2056581, EP 0285792 and US 5494805. These descriptions all deal with ready to use tests that make use of a test organism. The test organism is mostly imbedded in an agar medium, which may contain an indicator, a buffer solution, nutrients and substances to change the sensitivity for certain antimicrobial compounds in a positive or negative way.

Examples of suitable test organisms are strains of Bacillus, Streptococcus or E.coli. In general the principle of these tests is that when antibacterial compounds are present in a sample in a concentration sufficient to inhibit the growth of the test organism the color of an acid / base or redox indicator will remain the same, while when no inhibition occurs the growth of the test organism is accompanied by the formation of acid or reduced metabolites that will change the color of the indicator.

These test methods are suitable for the detection of antimicrobial residues in many food products. However up to now detection of antimicrobial residues in samples (e.g. some types of milk such as individual cow milk, liver, urine, kidney, meat juice, eggs), which contain a too high concentration of natural antimicrobial substances (e.g. lysozym, lactoferrine, lactoperoxidase) or a too high concentration of natural pigments (e.g. blood), was not easy to perform.

Said inhibiting substances show inhibitory activity against the microorganism of the test leading to false positive results (Okada et. al., Journal of the Japan Veterinary Medical Association 46: (2) 103 – 107 (1993); Schiffmann, Methodische und rechtliche Probleme beim Nachweis von Hemmstoffen in Milch, Publisher: Tierarztliche Hochschule, Hannover, Germany; Weisser, Tierarztliche Umschau 31: (6) 276 – 278 (1976); Heinert et. al., Archiv für Lebensmittelhygiene 27: (2) 55 – 60 (1976); Carlsson et. al., Milchwissenschaft 42: (5) 282 – 285 (1987); Carlsson et. al., Journal of Dairy Science 72: (12) 3166 – 3175 (1989)).

Natural inhibiting substances present in said samples can be inactivated by heating the sample, e.g. at 80°C for 10 minutes (Vermunt et. al., Netherlands Milk and Dairy Journal 47: (1) 31 – 40 (1993); Weisser, Tierarztliche Umschau 31: (6) 276 – 278 (1976)) or by using well known dialysis methods (Takahiro, Shokuhin Eiseigaku Zasshi 24: (4) 423 – 428 (1983); van Wall, Archiv für Lebensmittelhygiene 29: (6) 235 (1978)). After this pre-treatment the sample can be used for further testing by following the procedures of the test. In case of a microbial agar diffusion test (e.g. as described in EP 0005891) the liquid sample can be added directly to the test, after which the test is incubated.

Natural pigments (e.g. blood) present in the sample (e.g. meat juice or juice obtained from organs) will always interfere with the agar matrix. In case of tests based on a color shift using e.g. an acid / base or redox indicator, the presence of such natural pigments often leads to an unreadable test. A method to deminish the effect of the natural pigments is to execute a pre-incubation. In case of an agar diffusion test according to the method described in EP 0005891, the sample (e.g. meat fluid) is added to the test followed by a pre-incubation of e.g. 10 - 30 minutes at room temperature. This pre-incubation should be long enough to let the antimicrobial residues diffuse into the agar matrix. After the pre-incubation the sample is removed, the test is washed with water and incubated following the instructions of the producer. However said

methods require quite some extra time and handling and will not prevent diffusion of part of the disturbing compounds such as natural pigments into the agar. Even worse in case also a heating step (inactivation of natural inhibitors) is included always an ugly brown color appears, which make the test result even more unreadable. Mistakes by reading the results of the test may lead to both false positive and false negative results.

More over laboratories executing studies to the presence or absence of antimicrobial residues in foods are limited by the time available to execute these studies. With the present time consuming methods only a very limited amount of samples can be examined. Further these assays can only be executed in well-equipped laboratories and by well-educated persons, which is also a limited factor.

It can be concluded that up to now no suitable test methods for detecting antimicrobial residues in samples containing a too high concentration of natural inhibiting compounds and / or natural pigments are available. The present methods are time consuming and may lead to both false positive and false negative results, which leads to unacceptable amounts of antibiotics in the food chain and to economic losses.

Detailed description of the invention

The present invention now teaches that, reliable and very simple to carry out one step tests for the detection of antimicrobial residues in liquid samples which might contain high concentrations of natural inhibitors and / or disturbing compounds such as natural pigments are feasible.

According to one aspect of the invention it has been found that when such a sample is added to the test after which the test is incubated for a sufficient time at a sufficient temperature to inactivate the natural inhibiting compounds of the sample, the test can be incubated directly

after heating to determine the presence or absence of antimicrobial residues.

According to another aspect of the invention it has also been found that a suitable thickening agent can be added to the liquid sample, and disturbing compounds present in the sample can be catched in the matrix. Said matrix is preferably formed during the heating step.

It is even more surprising that antimicrobial residues diffuse directly from the solid matrix into the test system. Additional extraction methods to obtain the antimicrobial residues from the matrix are not required.

Surprisingly we also found that heating of the test containing the sample before incubation reduces the test duration considerably. This is probably caused by activation of the spores present in the agar matrix.

In a further aspect, the present invention provides a one step method for detecting antimicrobial residues in liquid samples, inactivation of the natural inhibiting substances of the sample and optionally catching disturbing compounds (e.g. blood) in a matrix using a thickening agent. The test can be executed using the following methods:

(1) Obtain a sample of the product to be tested, preferably a liquid sample, by using well known methods;

(2) Add a sufficient amount of the sample to the test using well known methods;

(3) Add optionally a suitable thickening agent in a suitable concentration to obtain a solid matrix, which is preferably formed during heating as described below;

(4) Heat the test, e.g. for approximately 10 minutes at 80° C, to inactivate the natural inhibiting substances (e.g. lysozyme);

(5) Incubate the test following the standard procedures of the test and read the result.

In case of conventional microbial inhibition tests (examples of commercial products are Delvotest®, Premi®Test, BR-Test®, the ADM

Copan® tests and the CHARM® AIM tests) inactivation of the natural inhibiting compounds present in the liquid sample, optionally formation of a matrix by adding a suitable thickening agent and activation of the spores of the test organism is preferably achieved by heating for 5 - 15 minutes at 75° C – 85°C. Alternatively any other temperature / time treatment, which is sufficient to obtain said effects, can be used. The exact requirements depend e.g. on the type of sample (milk, meat or organ juice, urine, egg, blood, etc.); the condition of the sample (e.g. the starting temperature, the volume of the sample); the type of test (e.g. microbial inhibition tests or assays based on selective binders (e.g. antibodies or tracers)); the microorganism used in the test (e.g. thermophilic or non-thermophilic Bacillus or Streptomyces species). Of course it should be take care of that the heat treatment will not inactivate the antimicrobial residues to be detected. The heat treatment can be executed using any method known in the art, e.g. by using an incubator as described in this invention or by heating in a water bath.

In a further aspect, the invention provides test units for carrying out the method of the invention. These test units contain the test and are suitable to execute the method of the invention: add the liquid sample, heat to inactivate the natural inhibiting compounds of the sample, optionally to form the matrix and activate the spores, incubate the test and read the results.

Examples of units useful for the purpose of the invention are transparent tubes, single or in a set or combined to a block of translucent material provided with a number of holes shaped therein. The test unit may contain solidified agar medium having therein said agar, optionally buffered; a test organism (e.g. a strain of Bacillus or Streptococcus) at sufficient colony forming units; nutrients for growth of said organism; an indicator (e.g. an acid-base or redox indicator); optionally substances to change the sensitivity for certain antimicrobial compounds in a positive or

negative way. All ingredients may optionally be added to the test as a separate source, for example as a tablet or paper disc.

The test units preferably have determined sizes. This is because of the reliability of the test. In case of a test based on agar diffusion technology preferably tubes are used. The test unit will preferably be high enough to contain an amount of agar medium and sample corresponding to a height of 3-30 mm, more preferably 5-15 mm. The internal cross-sectional dimension of the test units is preferably 1-30 mm, more preferably 5-15 mm. The test units are preferably closed air tight during storage in which conditions they may be stored for at least several months. Of course any other test unit suitable for executing the method of the invention is included in this invention.

The volume of the agar medium in the test unit is determined by the height of the test unit, the internal cross-sectional dimension of the test unit and the percentage of the volume of the test unit, which is filled with the agar medium. The volume of the agar medium is preferably 10 μl - 5 ml, more preferably 100 μl – 1 ml.

The invention also includes incubators suitable to execute the heat treatments as described in this invention. Incubators included in this invention are constructed in such a way that after placing the test units in the incubator, heat treatments as described in steps (4) and (5) (see above) can be done. The first heat treatment to inactivate the inhibiting compounds and optionally to form the solid matrix and / or to activate the spores is executed at a higher temperature, after which the incubation of the test continues at a lower temperature. Optionally after the incubation of the test the incubator can cool down to a temperature sufficient to stop the test.

An example of such an incubator is a block heater in which test units (e.g. ampoules) can be placed. For example in case of a conventional microbial agar diffusion test using a Bacillus stearothermophilus strain the incubator / block heater contains a number of holes suitable to place the test

ampoules or test plates (e.g. Delvotest® or Premi®Test). After placing the ampoules or plates the incubator heats the test to a temperature of e.g. 75°C – 85°C for e.g. 10 – 20 minutes after which the incubator turns to a lower temperature of 62°C – 65°C for 1.5 – 4 hours (incubation of the test). Of course the exact time / temperature intervals depend on many factors and will differ per type of test. This invention includes all incubators capable to execute a pre-incubation at a certain temperature for a certain period of time directly followed by an incubation at a lower temperature for a certain period of time. Optionally after the incubation of the test the incubator can cool down to a temperature sufficient to stop the test.

Any test suitable for the method of the invention is included in this invention. Examples are tests in which selected sensitive microorganisms are used, e.g. microbial agar diffusion tests, and tests based on selective binding of the compound to be detected. Selective binding can be achieved using the well-known antibody technology or by using specific tracers. An example of a specific tracer is the penicillin binding protein, which is used in e.g. the Delvo-X-Press® for detecting beta-lactams.

Examples of suitable microbial agar diffusion tests are tests in which species of Bacillus, Streptococcus or E. coli are used. Preferably thermophilic species, e.g. Bacillus stearothermophilus and Streptococcus thermophilus are used. Examples of preferred strains are Bacillus stearothermophilus var. calidolactis C953 (deposited with the Laboratory of Microbiology of the Technical University of Delft under the accession number LMD 74.1 in 1974 and with the Centraal Bureau voor Schimmelcultures (CBS), Baarn under the accession number CBS 760.83 in 1983 were the strain is available to the public) and Streptococcus thermophilus T101 (DSM 4022, deposited on March 3, 1987). Both strains are very sensitive to antimicrobial compounds, especially chemotherapeutics such as sulfa compounds and antibiotics such as

penicillins and tetracyclines. E.coli strains or other suitable gram-negative bacteria can be used for the detection of e.g. quinolones.

Bacillus stearothermophilus var. calidolactis C953 and Streptococcus thermophilus T101 are fast growing and have the advantage that they are thermophilic. For example the optimum growth temperature of said Bacillus strain is between 50° and 70°C. The test organism is therefore very suitable for a test according to the invention as it is not killed of by heating to inactivate the natural inhibiting compounds of the sample.

When the test organism is a Bacillus strain, it is preferably incorporated into the agar medium in the form of a spore suspension which may be prepared and incorporated into the agar medium prior to solidification by known methods (see for example Gb-A-1467439). When the test organism is a Streptococcus strain, the bacteria are preferably incorporated into the agar medium in the form of bacterial cells which may be prepared according to known methods (see for example EP 0285792). The concentration of the test organism in the agar medium is preferably between 105 and 1010 colony forming units per ml of agar medium.

Suitable nutrients to enable multiplication of the test organism in the absence of antimicrobial residues are for example assimilable carbon sources (e.g. lactose, glucose or dextrose), assimilable nitrogen sources (e.g. peptone) and sources of growth factors, vitamins and minerals (e.g. yeast extract).

The growth of the test microorganism can be detected using well known methods, preferably by color change of the agar medium of the test sample. Mostly a color indicator, preferably an acid-base or a redox indicator, is used. Examples of suitable acid-base indicators are bromocresolpurple and phenolred. Examples of suitable redox indicators are brilliant black, methylene blue, toludine blue and nile blue. Also combinations of two or more indicators can be used.

Optionally the sensitivity of the test may be altered by adding certain substances, by changing the test conditions such as pH or concentration of buffering substances or agar or by varying the ration of the volumes of agar and the sample. Examples of substances that may be added to the test system to change sensitivity are nucleosides such as adenosine and antifolates such as trimethoprim, ormethoprim and tetroxoprim, which improve the sensitivity of the test organism to sulfa compounds. Salts of oxalic acid or hydrofluoric acid may be added to improve the sensitivity to tetracyclines. Cysteine may be added to diminish the sensitivity to penicillins.

The amount of liquid sample to be added to the test depends on the test system. For microbial diffusion tests 0.01 – 1.0 ml, preferably 0.05 – 0.5 ml is added to the test using well-known methods.

Optionally suitable thickening agents can be added to the liquid sample. Examples of suitable thickening agents are polysaccharides (e.g. HMPC, locust bean gum, starch and xanthan) or proteins (e.g. egg albumin or whey proteins). Also combinations of suitable thickening agents can be used. The concentration of the suitable thickening agent should be sufficient to form a solid matrix, preferably in the temperature / time interval at which the first heating step is executed. If for example this step is executed by heating for 10 minutes at 80°C, e.g. to inactivate the natural inhibiting compounds, the concentration of the suitable thickening agent should be sufficient to form a solid matrix at said time / temperature conditions. Alternatively the solid matrix can also (partly) be formed during the incubation of the test. The thickening agent can be added to the liquid sample using any method known in the art, e.g. as a powder or as a tablet. The thickening agent can be added to the sample prior to addition of the sample to the test or after the sample is added to the test. Alternatively the thickening agent may be part of the test, e.g. as an ingredient of the agar or added onto the agar, e.g. as a tablet or as a powder, before the liquid sample is added to the test.

After addition of the sample the test is heated to inactivate the natural antimicrobial compounds and optionally to form a solid matrix and / or to activate the spores. Preferably the test is heated for 2 – 20 minutes at 70°C – 100°C, more preferably the test is heated for 10 - 15 minutes at 75°C – 85°C or for 2 – 6 minutes at 100°C. It is obvious that any other time / temperature treatment, which is sufficient to inactivate the natural inhibiting compounds of the sample without inactivating the antimicrobial residues to be detected, may be used.

After the heat treatment the test is incubated following the instructions of the producer. The incubation time of the test is dependent on the circumstances. In case of an agar diffusion tests using Bacillus stearothermophilus the test is incubated in a water bath or block heater at 60°C – 70°C, preferably at 62°C – to 65°C. Results may be obtained after 1.5 to 4 hours, preferably from 2.5 to 3.5 hours. In case of tests using selective binders, such as antibodies or tracers, the results may be obtained within 30 minutes.

The method described in this invention is very simple to carry out, so that persons who perform the test do not have to be specially educated.

All documents mentioned in this application are herein incorporated by reference to the same extent as if each individual application or patent was specifically and individually indicated to be incorporated by reference.

Example 1

Inactivation of natural inhibiting compounds present in a pre-urine sample

Fresh kidneys of 7 negative cows were obtained from a slaughterhouse (negative in the sence of the presence of antibiotics). To obtain samples for testing on the presence or absence of antimicrobial drug residues the rosettes of the kidneys were devided into pieces. The pieces were gently squeezed using a garlic press to obtain pre-urine.

Said samples were examined using microbial inhibition test ampoules produced according to the methods described in EP 0005891 with the nutrients present in the agar. Said test is also known as Premi®Test.

100 µl of each of the 7 squeezed samples (pre-urine) was added to the test ampoules (in triplo) and pre-incubated for 20 minutes at room temperature. It is known that this pre-incubation time is sufficient to let antimicrobial residues (if present in the sample) diffuse into the agar matrix of the test. After this pre-incubation the sample was removed using well known methods. Finally the test was incubated in a waterbath at 64°C using well known methods following the instructions of the producer. After 185 minutes incubation at 64°C all 3 samples of 4 animals were still positive (> 50%), After 200 minutes all 3 samples of 2 animals were still positive (> 30%). These false-positive results were caused by the inhibiting effect of natural inhibiting compounds present in the pre-urine.

100 µl of each of the 7 squeezed samples (pre-urine) was added to the test ampoules and heated for 10 minutes at 80°C in a waterbath, the ampoules were immediately placed in a waterbath of 64°C and incubated following the instructions of the producer. After 175 minutes the color of all tests turned from purple to yellow, indicating that no antimicrobial residues were present.

These results clearly demonstrate that natural inhibiting compounds of the pre-urine inhibit the test leading to false positive results. When the test is executed according to the method described in this invention by adding the sample directly to the test, heat the test as described above and incubate the test following the instructions of the producer, the activity of the natural inhibiting compounds is eliminated and no false-positive results are observed anymore.

Example 2

Inactivation of natural inhibiting compounds present in a pre-urine sample

This exeriment was executed according to the methods described in Example 1, except for the sampling procedure. In this experiment samples from the rosette of the kidney were ginded using well known methods. 100 µl of each of the 7 grinded samples (in triplo) was added to the test ampoules and pre-incubated for 20 minutes at room temperature, after which the samples were removed and the test was incubated as described in Example 1. After 220 minutes all 21 samples were still positive. More over due to a very ugly appearance of the test caused by a combination of the presence of natural pigments (e.g. blood) and heating of these pigment, it was not possible to read the results of the test in a proper way.

The experiment was repeated. However now the grinded samples were dilluted 1 : 1 with water. After 185 minutes still 3 samples were positive.

Finally the dilluted samples were also examined accoring to the method described in this invention. 100 µl of each of the 7 grinded dilluted samples was added to the test ampoules, heated for 10 minutes at 80°C and directly incubated as described in Example 1. After 175 minutes the color of all tests turned from purple to yellow, indicating that no antimicrobial residues were present.

These results clearly demonstrate that natural inhibiting compounds of the grinded kidney samples inhibit the test leading to false positive results. When the test is executed according to the method described in this invention by adding the sample directly to the test, heat the test as described above and incubate the test following the instructions of the producer, the activity of the natural inhibiting compounds is eliminated and no false-positive results are observed anymore.

Example 3

Inactivation of natural inhibiting compounds present in an egg sample

Samples of 5 eggs (in duplo), which did not contain antimicrobial residues, were obtained for examination on the presence or absence of antimicrobial residues. A hole of approximately 1-2 cm2 is made in the egg, the egg yolk is pricked and the egg is placed with the hole down on a bottle which allows the egg white and egg yolk to drip into the bottle. After the egg is empty, the bottle is closed and the sample is homogenized by shaking. Now the sample is ready for further studies.

To inactivate the natural inhibiting compounds present in the egg sample, 100 µl of each of the 5 samples was added on Delvotest® ampoules. The test was produced according to the methods described in EP 0005891 with the nutrients present in the agar. After heating for 10 minutes at 80°C in a waterbath, the ampoules were immediately placed in a waterbath at 64°C and incubated following the instructions of the producer. After 140 minutes the colour of all tests turned from purple to yellow, indicating that no antimicrobial residues were present.

The control samples were not heated at 80°C for 10 minutes, but directly placed on the ampoule. These tests remained purple for at least 4 hours.

These results clearly demonstrate that natural inhibiting compounds of the egg sample inhibit the test leading to false-positive results. When the sample is heated as described above, the activity of the natural inhibiting compounds is eliminated and no false-positive results are observed anymore.

Example 4

Determination of the sensitivity of the Delvotest® according to the method described in this invention using spiked samples

Egg samples were obtained according to the method described in Example 3. Said samples were spiked by adding Penicillin G (0 and 4 ppb) or Sulphadiazine (0 and 100 ppb) using well known methods. Said egg samples were added to Delvotest® ampoules (see Example II) according to the method described in this invention: heat for 10 minutes at 80°C, after which the ampoules are immediately placed in a waterbath at 64°C and incubated following the instructions of the producer. The results were read as soon as the blanco turned to yellow (after 140 minutes). The blanco samples were negative, while the samples spiked with 4 ppb Penicillin G and 100 ppb sulphadiazine remained purple (positive).

These results clearly demonstrate that the method descibed in this invention is suitable for detecting antimicrobial residues in egg samples.

Example 5

Use of a thickening agent in a kidney sample

Grinded kidney samples were obtained following the methods described in Example 2. However in this experiment the kidneys were first frozen. It is well known that after freezing of agrinded kidney sample so many inhibiting compounds come free, that up to now detecting antimicrobial residues using a microbial inhibition test was not possible.

A part of said sample was examined (five fold) using the methods described in Example 2. After 175 minutes all samples were positive, even after 190 minutes 3 samples were positive. More over the results were very difficult to read caused by the presence of natural pigments (e.g. blood).

An other part of said sample was examined as described above.However, now the thickening agent bovine albumine (Sigma) was added to the sample (6% and 8%, five fold) using well known methods, after which the sample was added to the tests and heated for 10 minutes at 80°C. After incubation of the tests for 175 minutes all 10 tests were negative, indicating that no antimicrobial residues were present. More over the purple and yellow colors of the test were very bright. Due to the presence of the matrix formed by adding bovine albumine both inhibiting compounds and natural pigments are catched in the matrix.

These results clearly demonstrate that adding a thickening agent to the grinded and froozen kidney sample makes it possible to execute the test in a proper way. Both natural inhibiting compounds and natural pigments are catched in the matrix.

CLAIMS

1. A process to detect antimicrobial residues in a liquid sample comprising:
   - taking a sufficient amount of a sample to be tested,
   - adding the sample on a test suitable to detect antimicrobial residues,
   - treating the test and sample for a sufficient time interval to inactivate natural inhibiting compounds present in the sample,
   - incubating the test, and
   - determining the presence of antimicrobial residues.

2. A process according to claim 1 whereby a thickening agent is added to the sample to form a solid matrix of said sample.

3. A test unit which comprises
   - a test for the detection of antimicrobial residues, and
   - a sample added to the test, whereby the compounds present in the sample, which inhibit the test, are inactivated.

4. A test unit according to claim 3 which comprises a sample to which a thickening agent is added.

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A NEW TEST METHOD FOR THE DETECTION OF ANTIMICROBIAL RESIDUES IN LIQUIDS

ABSTRACT

The present invention relates to a novel method for the rapid detection of the presence or absence of antimicrobial residues in products preferably food products.